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We have fully characterized the expression and localization of claudin 7 in the mammary epithelium, finding that it is not associated with tight junctions but is constitutively present in punctate structures, presumably vesicles, at the basal and lateral borders of cells. We find the same to be true of mouse mammary tumors. We find claudins 1, 3, 4, 5 and 8 to be present in at least one of our mouse mammary cell lines, where they are localized to the tight junction. Only claudin 1 has been found to be consistently present in mammary epithelial tight junctions. Claudin 3 is localized to the basal and lateral borders of the cells in the virgin and pregnant animals, but not observed in lactation. Claudin 8 is prominent at the tight junctions in the mammary epithelium from the lactating animal. In the next year we will complete these analyses and apply them to mouse mammary tumors. In addition we plan to use our adenoviral technology to overexpress claudin 8 during pregnancy to determine whether it can contribute to closure of the tight junctions under conditions where the junctions are normally open.

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INTRODUCTION

The purpose of the research funded under the above research grant was to examine the hypothesis that the gene for the tight junction protein, claudin-7, plays a role in breast cancer. We planned to test the hypothesis that high expression of claudin-7 can signify one or more novel roles in tumorigenesis by:

       1. Determining the cellular and subcellular localization of claudin-7 in the mouse mammary gland.

       2. Comparing mRNA levels of claudin-7 to claudin-1, claudin-3 and two adherens proteins, E-cadherin and beta-catenin.

       3. Examining the functional significance of claudin-7 by overexpressing it in cell lines and in the mammary gland.

Objective 1, which comprises TASKS 1, 2 and 3 in the statement of work was completed as of the last annual report. We have, however, refined our immunocytochemical analysis to show that claudin 7 is not only a constitutive component of the mammary luminal epithelium but is present solely at the basal-lateral borders of the cell and does not overlap the tight junctions. It appears both to be present in the membranes and in vesicles near the membrane. These data are currently being written up for publication.

Objective 2, which comprises parts of tasks 5 and 6 was largely completed based on microarray analysis last year. However, we have been examining claudins 1-6 and 8 in more detail, using antibodies to obtain localization of these molecules. Details of the localization of some of these claudins will be presented in this report

Objective 3 was changed to examine the functional significance of claudins. Because we have found significant changes in claudins 3 and 8, we have cloned these molecules and plan to use them in expression studies in the normal gland. Once we have verified that the antibodies we have obtained are indeed specific for these other claudins, we will examine a panel of mouse mammary tumors to determine whether there are claudins whose expression is altered at the protein level in the tumor state.

We have spent most of the year determining the specificity of the available claudin antibodies and have found this to be a particularly difficult task, as many of the antibodies available, particularly those from Santa Cruz, do not work as advertised. The criteria we use for antibody specificity are:

1. The antibody shows localization to the tissue in which it has been demonstrated by previous investigators and does not stain tissues that have been shown to be negative by immunohistochemistry or Northern analysis.

2. Localization to tight junctions by immunohistochemistry in mouse mammary cell lines that form tight junctions in vivo, either EPH4 cells or CIT3 cells.

3. Blocking by incubation with the specific peptide against which the antibody has been made.

We will start the report by demonstrating the results of our continuing studies on claudin-7 and then summarize our progress with the other claudins in the 1 to 8 series.

BODY:

1. Claudin-7 localization in the mammary epithelial cell.

We had shown last year that claudin-7 is a constitutive component of mammary epithelial cells at all stages of mammary development and

Figure 1. Mouse epididymis stained with antibodies to claudin 7 and the tight junction protein ZO-1. Note the overlap between Claudin 7 and ZO1 staining in the merged image (Yellow).

that it was also present in mammary tumors. We had characterized an antibody and shown that it cross reacts with the protein on Western blots (TASK 4), stains only the luminal epithelium in sections of mouse mammary gland, stains tight junctions in EPH4 cells (Figure 5) and reacts with lung epithelial cells, where the mRNA can be detected (1) but not with cells in the liver where it is not present. It stains both frozen and paraffin sections. Staining is blocked by the peptide against which the antibody was made and is not seen in the absence of antibody. All these criteria indicate that the antibody is specific for claudin-7. This year we have additional data showing that the protein does localize to tight junctions the epididymus (Figure 1). Note that the stain in the upper panel coincides at least in part with the cobblestone stain in the second panel indicating overlap at the tight junctions in this tissue, where there also appears to be cytoplasmic stain. However, Claudin 7 appears to be completely excluded from the tight junctions of mammary epithelial cells as shown by the data in Figures 2 and 3. The higher power view in Figure 3 shows that the stain is punctate in nature suggesting a vesicular localization near the borders of the cell. This significance of this localization is not known at this point. It is however, one of very few documented instances where the claudin doesn't localize to the tight junction.

Figure 2. Section of a mammary gland from a pregnant mouse showing complete segregation of green (ZO-1) and red (claudin 7) stain. The nuclei are stained blue with DAPI.
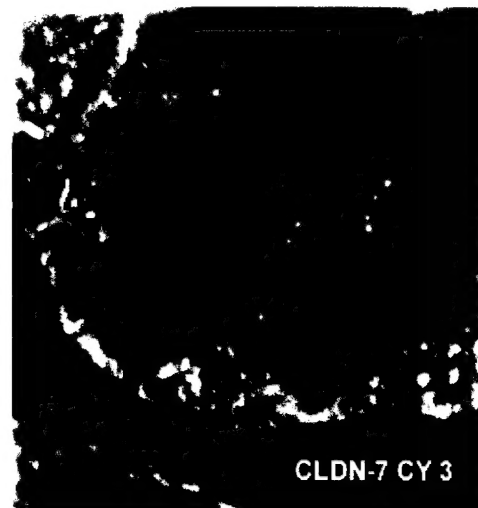

Figure 3. High power section of the basal portion of an epithelial cell from a lactating gland showing the punctate nature of the claudin-7 stain.

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˙2. Claudin expression in the mammary epithelium—microarray analysis.

We have expanded our microarray analysis of mammary gland claudins during normal development. Figure 4 shows the expression of claudins 1 through 8 in the mammary epithelium in a developmental series that used 4 animals for every point compared to GAPDH expression. Claudin 3 increases through pregnancy dropping off sharply with the onset of lactation. Claudin 8, on the other hand, is highly expressed during pregnancy. Claudins 4 and 5 also appear to be developmentally regulated. Claudins 1, 2 and 6 are expressed at very low levels and we have, of course, already described the expression of claudin 7 (above and last years progress report). We procured antibodies to all these claudins to determine whether the protein was expressed and its localization. Table 1 and the paragraphs below summarize our progress in this regard.
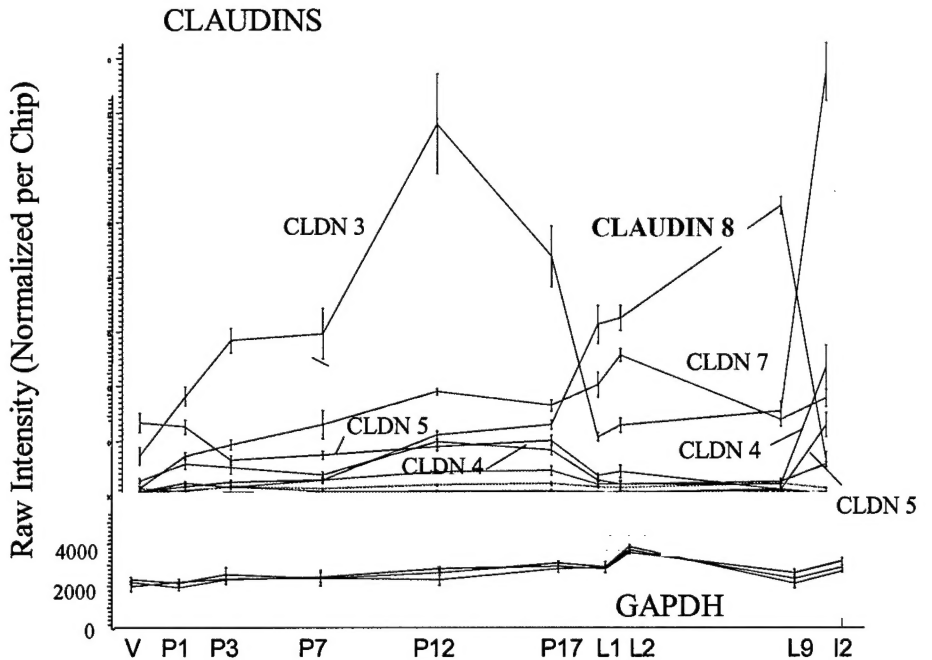

Figure 4. Microarray data for developmental regulation of claudin expression in the mammary gland from the virgin (V) through pregnancy days 1, 3, 7, 12 and 17, lactation days 1, 2 and 9 and two days of forced involution after pup removal at day 9 (I2).

3. Immunohistochemical staining for Claudins 1-6 and 8 in the mammary gland.

Table 1 summarizes our progress to date. At this point we are missing some controls. The results will by summarized by claudin. For all antibodies we have examined staining in EPH4 and CIT3 lines of mouse mammary epithelial cells. In addition we have generally examined tissues reported to be either positive or negative for the claudin, although this analysis is not complete. We have also examined staining in the mouse mammary gland in virgin, pregnant and lactating animals. Where we found stain associated with the tight junctions we have or will procure a blocking peptide to further test the specificity of the stain. We show only selected results with Claudins 1, 3 and 8.

Claudin 1. Although claudin 1 gene expression is low we did find claudin 1 localized to tight junctions at all stages of development. Figure 5 shows results from the virgin mouse, but similar observations were made in pregnancy and lactation. Interestingly the stain is concentrated at the junction but extends beyond the junction in a way that suggests that it may be trafficking to or from the junction in vesicles. This was a common finding in all preparations where claudins were observed at tight junctions.

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Table 1: Immunohistochemistry of claudins 1 through 8 through mammary development

	QRT1	Array	peptide block	Tissue culture	Positive control	Negative control	Virgin	Pregnancy	Lactation
Claudin 1	TBD	present, low	YES, OK	YES	mammary gland	Blood vessels	All stages--colocalizes with ZO1 at tight junctions		
Claudin 2	TBD	Absent, very low	NO	Not Seen	TDB	EPH4 cells	No expression at TJ		
Claudin 3	TBD	Down at Lac	No peptide	YES, EPH-4 at junctions	TBD	TBD	Not at junctions, punctate in cytoplasm		None
Claudin 4	TBD	Present preg; Absent lactation; high involution	NO	CIT3-yes	EPH4--yes	TBD	Nothing at TJ, but possible interstitial stain (blood vessels?)		
Claudin 5	TBD	Present preg; Absent lactation; high involution	NO	CIT3 yes, EPH4, yes	TBD	NO	Vague diffuse apical stain; interstitial stain		
Claudin 6	TBD	Absent		No stain	TBD	NO	Background only		
Claudin 7	Yes	Expression constant	Yes	Yes	Epididymis	Liver	Expressed at all stages, but localized basally and laterally		
Claudin 8	Yes	Up at Lac	Yes	no-EPH4; yes;CIT3	Kidney	Heart-no stain	Not at TJ	Apical, some TJ, not all	Clear stain at tight junctions

TBD, to be done; Lac, lactation; Preg, pregnant; EPH4 and CIT3, mouse mammary cell lines; TJ, tight junction
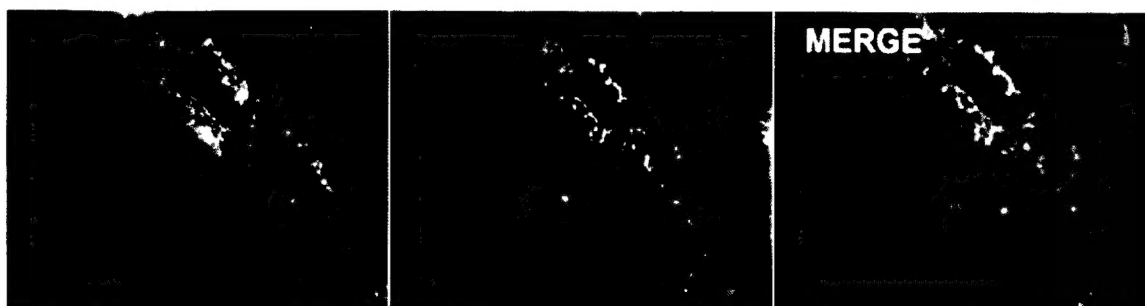
Figure 5. Stain for Claudin 1 (red), ZO1 (green) and nuclei (blue) in a frozen section from mammary duct from a virgin mouse. The lattice work at the apical borders of the cells stains for both claudin 1 and ZO1 as shown clearly in the black and white versions that show each fluor separately.

Claudin 2. We were unable to find stain for claudin 2 in any of the mammary tissues or cell lines observed. We have not yet verified the positive control for this claudin so it may be that the antibody is not working on our sections.

Claudin 3. Claudin 3 gene expression increases markedly at mid pregnancy with a sharp decline at the onset of lactation. Although claudin 3 stained tight junctions in EPH4 cells (data not shown) it was distributed at the basal and lateral borders of mammary epithelial cells from virgin and pregnant mice. Figure 6 shows that this claudin is distributed at tight junctions in EPH4
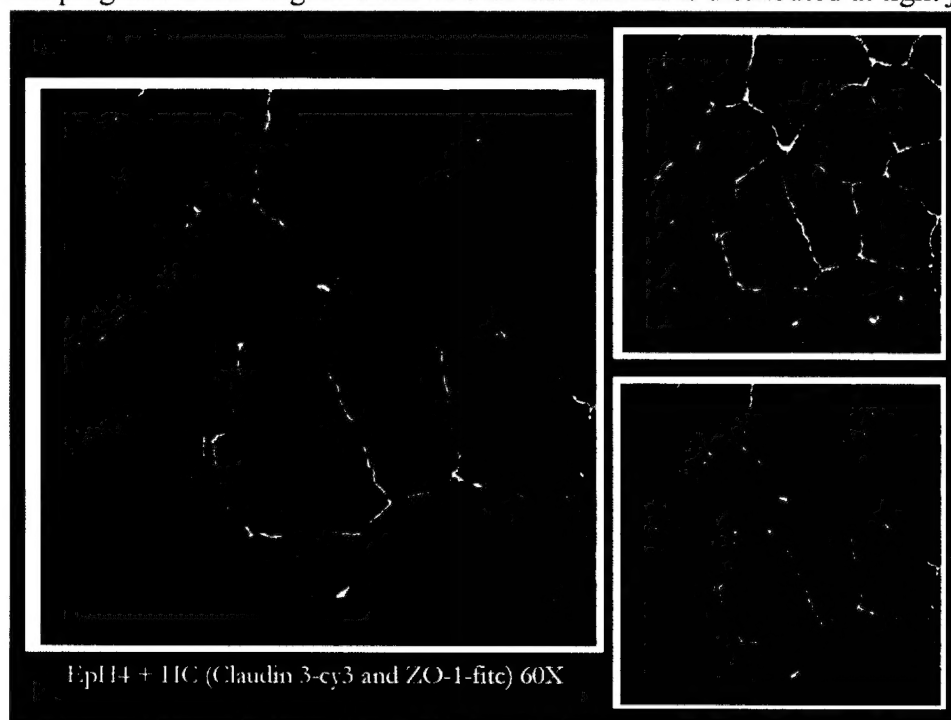

Figure 6. Claudin 3 stain in EPH4 cells. Claudin 3 (red) and ZO1 (green) are merged in this image from of cultured cells taken with a digital confocal microscope.

cells suggesting that the antibody does indeed stain for a claudin. Figure 7 shows a paraffin section of a mammary gland from a pregnant mouse. ZO1 (green) shows a clear lattice work at the apical borders of the cell but claudin 3 stain (red) does not overlap with ZO-1 but is confined to the cytoplasm. No claudin 3 stain was observed in lactating mice. The implications of its cytosolic expression in pregancy are not clear. It will be of interest to determine whether this pattern is repeated in mammary tumors as it is for claudin 7.
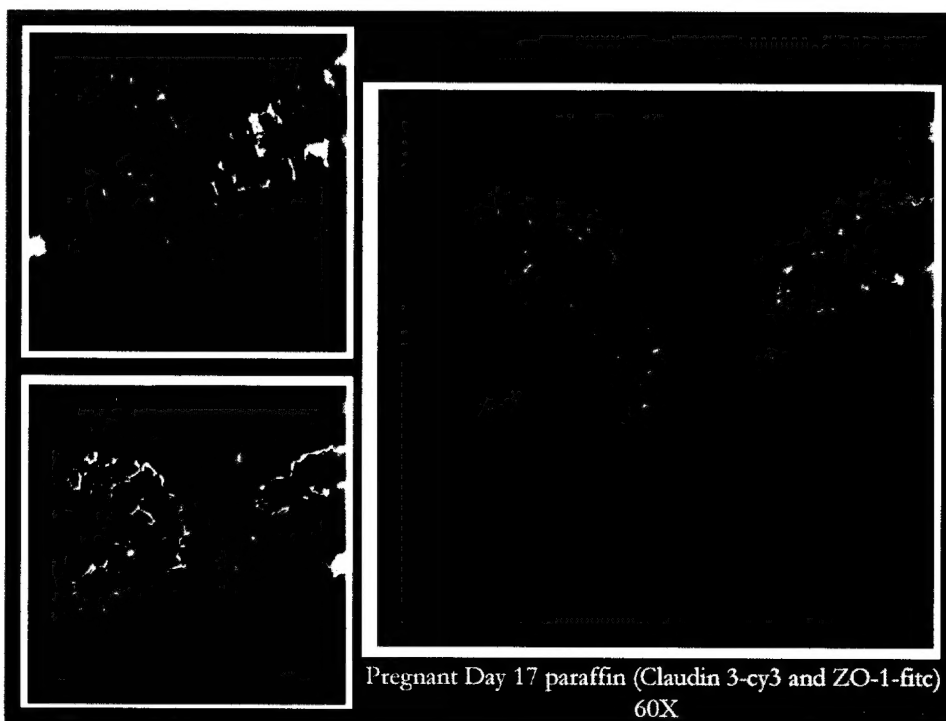
Figure 7. Claudin 3 and ZO1 staining of a paraffin section from a mammary gland of a pregnant mouse. Stains for the two probes do not overlap. In addition Claudin 3 stain is excluded from the nucleus.

Claudin 4. From the array data, claudin 4 was present at pregnancy but absent at lactation, increasing again at involution in mammary tissue. The antibody showed positive staining at tight junctions in both EPH4 and CIT3 cells, However, there was no stain in the mammary epithelium although there appeared to be some interstitial stain which could represent blood vessels.

Claudin 5. Gene expression was low in pregnancy and lactation but increased at involution. Tight junction stain was observed in both EPH4 and CIT3 cells, but no stain was observed in either the pregnant or lactating gland. We have not yet examined involution.

Claudin 6. Gene expression was very low at all stages of mammary development and we were unable to detect any stain in either cultured cells or mammary epithelium. The positive control has yet to be done for this claudin and the antibody may be suspect because it came from Santa Cruz. The remainder of our antibodies are from Zymed, a more reliable company.

Claudin 8. Claudin 8 is the most interesting of the claudins examined to this point. Its gene expression is low through pregnancy with a sharp increase at lactation. We detected the protein, using a purpose made antibody from Zymed, at the tight junctions of CIT3 cells but not in EPH4 cells. We did not detect stain in virgin tissue. Occasional tight junctions were stained in the pregnant animal, whereas stain was consistently observed at tight junctions in the lactating animal (Figure 8). This finding is exciting because claudin 8 has been associated with reduced permeability in cultures of MDCK cells (1). We have previously reported that claudin 8 mRNA is present in mammary tumors, but we have not yet examined these tumors for the presence and localization of the protein.
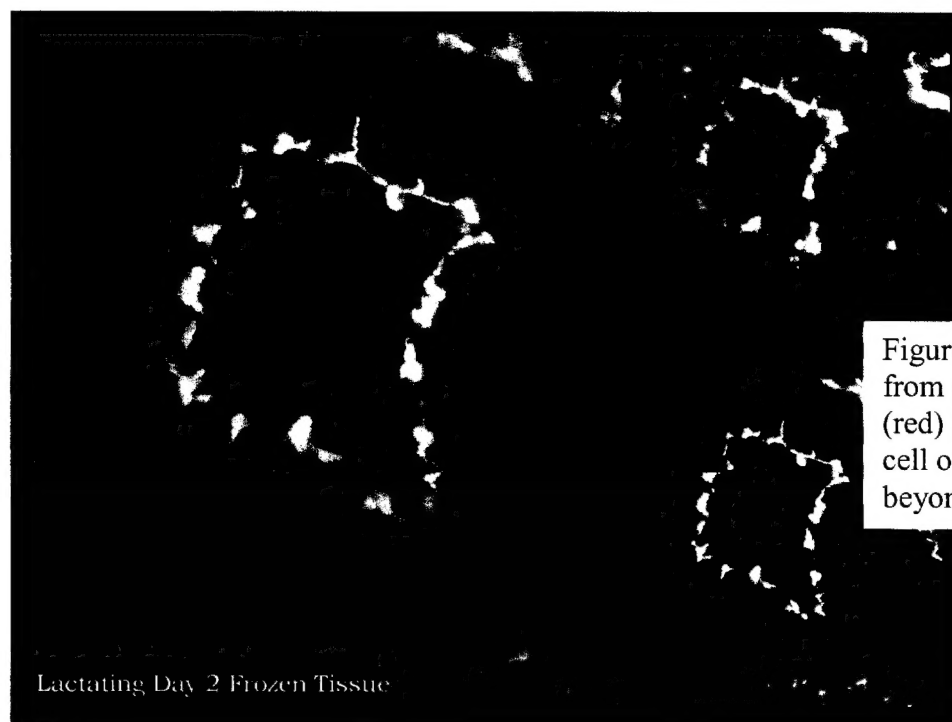
Figure 8. Claudin 8 staining in a section from day 2 lactating tissue. Claudin 8 (red) is found at the apical borders of the cell overlapping with and extending beyond ZO-1 stain.

KEY RESEARCH ACCOMPLISHMENTS since the beginning of the grant. New findings are in red:

- A specific and highly active antibody to claudin 7 has been developed and shown to react with claudin 7 on Western blots (tasks 1and 4).
- Claudin-7 is localized to luminal epithelial cells in the mammary gland throughout development by *in situ* hybridization and specifically to the basolateral membranes of these cells in both normal glands and mammary tumors as shown by immunohistochemistry (Task 2). This claudin is present at tight junctions in cultured cells and epidymus, but not in lung or mammary epithelium.
- The developmental expression of claudin-7 mRNA was shown to parallel expression of keratin by real time RT-PCR and high expression was found in a series of tumors (Task 3).
- Expression of additional claudins and claudin-like molecules has been quantitated by microarray analysis using Affymetrix arrays (Task 5). The expression of claudin 8 has been confirmed by real time RT-PCR and found to increase more than 10 fold at lactation. The protein was found in the tight junctions of the mammary epithelium only in the lactating gland.
- Claudin 8 is expressed in mammary tumors although they lack tight junctions. (Task 5).
- Claudins 1 has been localized to tight junctions in mammary epithelial tissues throughout development (Task 5).
- Claudins 3, 4, 5, and 8 were observed at tight junctions in mammary cell lines. However, claudin 8 was the only one of these associated with the mammary tissue tight junction, and then only at lactation.
- Claudins 2 and 6 were not observed in any mammary epithelium.

REPORTABLE OUTCOMES.

A poster detailing our findings with claudin 7 was presented at the Era of Hope meeting in Orlando, Fl in October, 2002.

A publication is underway to describe the results with claudin 7.

A paper describing the use of adenoviral transduction to express foreign genes in the mammary epithelium has been published in J. Virol. (Appendix).

CONCLUSIONS.

We have now fully characterized the expression and localization of claudin 7 in the mammary epithelium, finding that it is not associated with tight junctions but is constitutively present in punctate structures, presumably vesicles, at the basal and lateral borders of cells. We find the same to be true of mouse mammary tumors. We are now in the process of fully characterizing the expression of claudins 1 through 6 and claudin 8 in the developing mammary epithelium and in mammary cell lines. We find claudins 1, 3, 4, 5 and 8 to be present in at least one of our mouse mammary cell lines, where they are localized to the tight junction. However, only claudin 1 has been found to be consistently present in mammary epithelial tight junctions. Claudin 3 is localized to the basal and lateral borders of the cells in the virgin and pregnant animals, but not observed in lactation. Claudin 8 is prominent at the tight junctions in the mammary epithelium from the lactating animal but not at other developmental stages suggesting it may participate in the closure of tight junctions observed during lactation.
In the next year we plan to complete these analyses, to apply them to mouse mammary tumors and to examine the expression of additional claudins as reliable antibodies become available. In addition we plan to use our adenoviral technology to overexpress claudin 8 during pregnancy to determine whether it can contribute to closure of the tight junctions under conditions where the junctions are normally open.

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APPENDIX

Russell T, Fischer A, Beeman N, Freed E, Neville MC, Schaack J. Transduction of the mouse mammary epithelium with adenoviral vectors *in vivo*. J Virol 2003;77:5801-9.

Transduction of the Mammary Epithelium with Adenovirus Vectors In Vivo

Tanya D. Russell,[1] Andreas Fischer,[1] Neal E. Beeman,[1] Emily F. Freed,[1] Margaret C. Neville,[1] and Jerome Schaack[2]*

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Because the mammary parenchyma is accessible from the exterior of an animal through the mammary duct, adenovirus transduction holds promise for the short-term delivery of genes to the mammary epithelium for both research and therapeutic purposes. To optimize the procedure and evaluate its efficacy, an adenovirus vector (human adenovirus type 5) encoding a green fluorescent protein (GFP) reporter and deleted of E1 and E3 was injected intraductally into the mouse mammary gland. We evaluated induction of inflammation (by intraductal injection of [^{14}C]sucrose and histological examination), efficiency of transduction, and maintenance of normal function in transduced cells. We found that transduction of the total epithelium in the proximal portion of the third mammary gland varied from 7% to 25% at a dose of 2×10^6 PFU of adenovirus injected into day 17 pregnant mice. Transduction was maintained for at least 7 days with minimal inflammatory response; however, significant mastitis was observed 12 days after transduction. Adenovirus transduction could also be used in the virgin animal with little mastitis 3 days after transduction. Transduced mammary epithelial cells maintained normal morphology and function. Our results demonstrate that intraductal injection of adenovirus vectors provides a versatile and noninvasive method of investigating genes of interest in mouse mammary epithelial cells.

The mammary gland is a compound lobulotubular structure that is a reliable model for developmental studies of cellular growth and differentiation, epithelium-stroma interactions, and tissue-level analysis of systemic hormonal regulation (13). In humans, it begins to develop in the 18- to 19-week fetus and after birth remains quiescent until puberty, when hormonal stimulus by estrogen and growth hormone triggers the tree-like branching of a network of ducts that extends from the nipple into the mammary fat pad on the anterior wall of the thorax (12, 13). Lobular structures, which will become the milk-secreting acini, originate from these ducts. Lobular development is highly dependent upon hormonal stimulation, and in humans, in whom these lobular structures are known as terminal duct lobular units, begins after the onset of menses. In mice the extent of lobular development in the virgin animal is strain dependent (12). Full alveolar development and maturation of the mammary epithelium take place during pregnancy and are dependent upon high circulating concentrations of progesterone, prolactin, and/or placental lactogen (12). Upon withdrawal of progesterone at parturition, lactation commences. Ongoing milk secretion continues during lactation under the influence of prolactin and oxytocin and ceases at weaning. When regular extraction of milk ceases, the alveolar epithelium undergoes apoptosis and remodeling, and the gland reverts to a prepubertal stage (12).

Since the mammary gland undergoes its functional morphogenesis in the young adult to adult stages and is very suscep-

tible to tumorigenesis (13), it would be desirable to be able to manipulate its genetic complement at different developmental stages and study the effects of these changes. For this reason, we investigated the utility of adenovirus transduction in vivo to alter gene expression by using injection through the nipple to gain access to the epithelium from the exterior of the animal. Earlier investigators have taken advantage of intraductal injection techniques, using injections in goats and mice to study the permeability of the mammary epithelium to Na$^+$, Cl$^-$, and radiolabeled sucrose (8, 16).

DEAE-dextran-mediated transfection has been used to obtain human growth hormone expression in the guinea pig mammary gland after intraductal injection (5). However, the proportion of transfected cells was quite low. On the other hand, adenovirus transduction has proven to be a suitable method for efficient transduction of primary mammary cells in vitro in combination with mammary gland reconstitution to yield highly efficient gene transfer (18). In vivo, Jeng and coworkers injected an adenovirus vector coding for β-galactosidase into the rat mammary gland through the mammary duct and obtained significant expression of the gene (6). Yang et al. (24) obtained expression of LacZ in the mouse mammary gland in vivo after intraductal injection of an adenovirus vector. Although these studies demonstrated the effectiveness of adenovirus vectors, the issues of an inflammatory response and the efficiency of transduction have not been fully addressed.

We are primarily interested in the transition from pregnancy to lactation and sought a noninvasive, noninflammatory delivery system for introducing foreign genes into the mammary epithelium without transduction of the surrounding stroma. Our laboratory has perfected a technique of intraductal microinjection into the mouse mammary gland (14) and has used this

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technique to analyze tight junction regulation (15) and tight junction permeability relative to progesterone withdrawal and the presence of glucocorticoids (16) in the late pregnant mouse. We hypothesized that a similar intraductal injection technique could be used to obtain direct and localized transduction of the mammary epithelium with adenovirus vectors with minimal inflammation and little stress to the animal. The present study demonstrates that intraductal injection of a green fluorescent protein (GFP)-encoding adenovirus vector at late pregnancy leads to successful transduction of the epithelial cells in the proximal portion of the gland that lasts through parturition and into at least 5 days of lactation without inflammation. Although we examined transduction at late pregnancy most carefully, we also present data obtained at other stages of mammary gland development.

MATERIALS AND METHODS

Animals. CD-1 mice aged 5 to 9 weeks were purchased from Charles River Breeding Laboratory (Wilmington, Mass.) and maintained in the U.S. Department of Agriculture-approved Center for Laboratory Animal Care of the University of Colorado Health Sciences Center Animal Care Facility. Nulliparous, early pregnant (3 days; P3), late pregnant (17 days; P17), and lactating (4 days postparturition; L4) mice were used in this study. Pregnancies were timed by observing a vaginal plug (day 1 of pregnancy) after overnight residence with a male. The due date was calculated as 19 days after observation of the vaginal plug. Mice were housed under a 12-h light–12-h dark cycle and maintained on either breeder's chow (Teklad S-2335, no. 7004 mouse breeder diet; Harlan Teklad, Madison, Wis.) or a standard diet for postbreeding females (Teklad 22/5, no. 8640 rodent diet; Harlan Teklad, Madison, Wis.) and tap water ad libitum. All mice were anesthetized by intraperitoneal injection with avertin (125 to 250 mg/kg) and sacrificed by cervical dislocation. All procedures were approved by the Internal Animal Care and Use Committee of the University of Colorado.

Adenovirus vectors. Adenovirus vectors were grown in 293 cells, which are transformed by and express high levels of the adenovirus type 5 E1A and E1B proteins (4). A replication-defective adenovirus type 5 vector encoding enhanced, humanized, red-shifted green fluorescent protein under the control of the human cytomegalovirus major immediate-early promoter (Ad5GFP) was described previously (19).

Virus growth. Viruses were grown in 293 cells in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 10% bovine calf serum. For growth of high-titer stocks, 293 cells were infected and harvested by centrifugation at the time of maximal cytopathic effect, and the virus was released by three cycles of freezing and thawing. Cell debris was pelleted, the supernatant was saved, and the pellet was resuspended in phosphate-buffered saline (PBS), frozen and thawed, and pelleted. The supernatant was combined with the first supernatant. The pellet was resuspended in PBS and pelleted. The supernatant was combined with the prior supernatants. The supernatants were overlaid on a step gradient consisting of 1.25 and 1.4 g of CsCl per ml in PBS and centrifuged for 50 min at 36,000 rpm in an SW41 rotor (Beckman). The virus band was collected by side puncture, diluted with 1.35 g of CsCl per ml in PBS, and centrifuged for 3 h at 65,000 rpm in a VTi65 rotor (Beckman). The virus band was collected by side puncture, dialyzed for 1 h each against three changes of adenovirus storage buffer (10 mM Tris-HCl [pH 8.0], 135 mM NaCl, 1 mM MgCl$_2$, 50% [vol/vol] glycerol), and stored at $-20°C$ until use. The concentration of virus particles was determined from the absorption at 260 nm, with 1 A_{260} unit being equivalent to 10^{12} particles. Virus stocks were plaque titered on 293 cells.

Adenovirus microinjection. Ad5GFP microinjection was performed under avertin anesthesia at various stages of mammary gland development (Table 1). A stock of 2.7×10^8 PFU/ml was made in adenovirus storage buffer. Final doses (2.7×10^7 PFU for fourth mammary glands; 2×10^6 PFU for third mammary glands) were made by diluting the 2.7×10^8 PFU/ml stock with sterile filtered Ringer's solution (138 mM NaCl, 8.1 mM Na$_2$HPO$_4$, 1.2 mM K$_2$HPO$_4$, 2.7 mM KH$_2$PO$_4$, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$). This dilution was made immediately before the microinjection to ensure the stability of the adenovirus. The solution was loaded into a 25-μl Wiretrol II disposable glass micropipette with a stainless steel plunger (no. 5-000-2050; Drummond Scientific Company, Broomall, Pa.). The end was drawn and fire-polished into a fine tip of 60 to 75 μm. By using a micromanipulator, the tip was gently inserted into the teat canal, and the solution was slowly ejected into the lumen of the either the third or fourth mammary

TABLE 1. Experimental design

Reproductive stage		Total microinjection vol (μl)
Injection	End point[a]	
Nulliparous	3	5
P3	P6	80
P17	L2	80
P17	L5	80
P17	L10	80
P17	L11	80
P17	L15	80
P17	L27	80
L4	L7	80

[a] Days after injection, pregnancy day, or lactation day.

gland as previously described (14). To evaluate the reliability of the injection technique, sterile filtered Ringer's solution was injected into contralateral control glands in some experiments.

Determination of mammary epithelium permeability. Since increased [^{14}C]sucrose permeability is one of the hallmarks of mastitis (9), we used this isotope to determine mammary epithelium permeability. On the day of sacrifice, 2 μCi of [^{14}C]sucrose (Amersham, Buckinghamshire, United Kingdom) were lyophilized and dissolved in sterile Ringer's solution. Then 5 μl (nulliparous glands), 40 μl (fourth mammary glands), or 20 μl (third mammary glands) of this solution was injected intraductally into the lumens of adenovirus-injected and contralateral control mammary glands of each mouse under avertin anesthesia. The 10-μl blood samples were taken from the tail vein 5 min after each injection, and the amount of ^{14}C present was determined by liquid scintillation counting.

Preparation of tissue for freezing and histology. Injected experimental and contralateral control glands were excised and cut horizontally in half. One half of the gland was fixed in formalin, embedded in paraffin, and cut and stained with hematoxylin and eosin for histological purposes. The other half was cut into four to six smaller pieces and placed in aluminum foil molds filled with embedding medium (Tissue-Tek O.C.T. compound no. 4583; Sakura Finetek U.S.A., Inc, Torrance, Calif.) for frozen tissue specimens. The molds were flash frozen by immersion in an isopentane bath brought to its cooling point with liquid nitrogen.

Frozen sectioning and immunohistochemistry. Coverslips (Fisher Scientific no. 12-544-10) were treated with BD Cell-Tak and tissue adhesive (BD Biosciences no. 354240), rinsed, and stored overnight at 4°C. Then 12-μm sections were cut from the frozen molds with a cryostat at $-32°C$ and collected onto the treated coverslips. The samples were placed at 37°C for 1 h and fixed in 2% paraformaldehyde (no. 00380; Polysciences, Inc., Warrington, Pa.) for 10 min.

After rinsing two to three times with PBS, the samples were treated with a blocking solution of 5% normal goat serum (#005-000-121; Jackson Immunoresearch, West Grove, Pa.) and 100 μg of saponin per ml (no. S4521; Sigma, St. Louis, Mo.). Samples were rinsed twice with PBS and incubated with the appropriate primary antibody for 1 h. A polyclonal antibody (7781) was made by using casein precipitated at pH 6.3 from mouse milk. Western blots showed specificity for mouse β-casein. Antibody against xanthine oxidase was generated against purified mouse xanthine oxidase in rabbit and purified on protein A-Sepharose (10). For nonantibody staining, samples were treated with wheat germ agglutinin conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) to outline the luminal surface of mammary epithelial cells, and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma D-9542) diluted in PBS was used to stain for nuclei.

Samples incubated with a primary antibody were rinsed five times for 5 min each with PBS and treated with both donkey anti-rabbit IgG conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) and DAPI diluted in PBS. Both antibody-treated and non-antibody-treated samples were then rinsed six times for 5 min each in PBS. Then 60 μl of mounting medium (ProLong antifade kit, no. P-7481; Molecular Probes, Eugene, Oreg.) were placed on slides (Fisherbrand Superfrost, no. 12-550-15; Fisher Scientific, Pittsburgh, Pa.), and the coverslips were carefully lowered onto each slide. The slides were kept in the dark overnight and then placed at 4°C for storage.

Determination of mastitis. We developed a mastitis scoring system to examine the inflammatory response in Ad5GFP-transduced mammary epithelium. Three randomly chosen fields from hematoxylin and eosin-stained slides were assessed by bright-field microscopy at 40× magnification from various samples for the number of polymorphonuclear cells, mononuclear cell infiltration (scored 0, 1, and 2), and epithelial organization, again on a subjective scale (scored 0, 1, and 2), where 1 represents some mononuclear cell infiltration and epithelial disor-
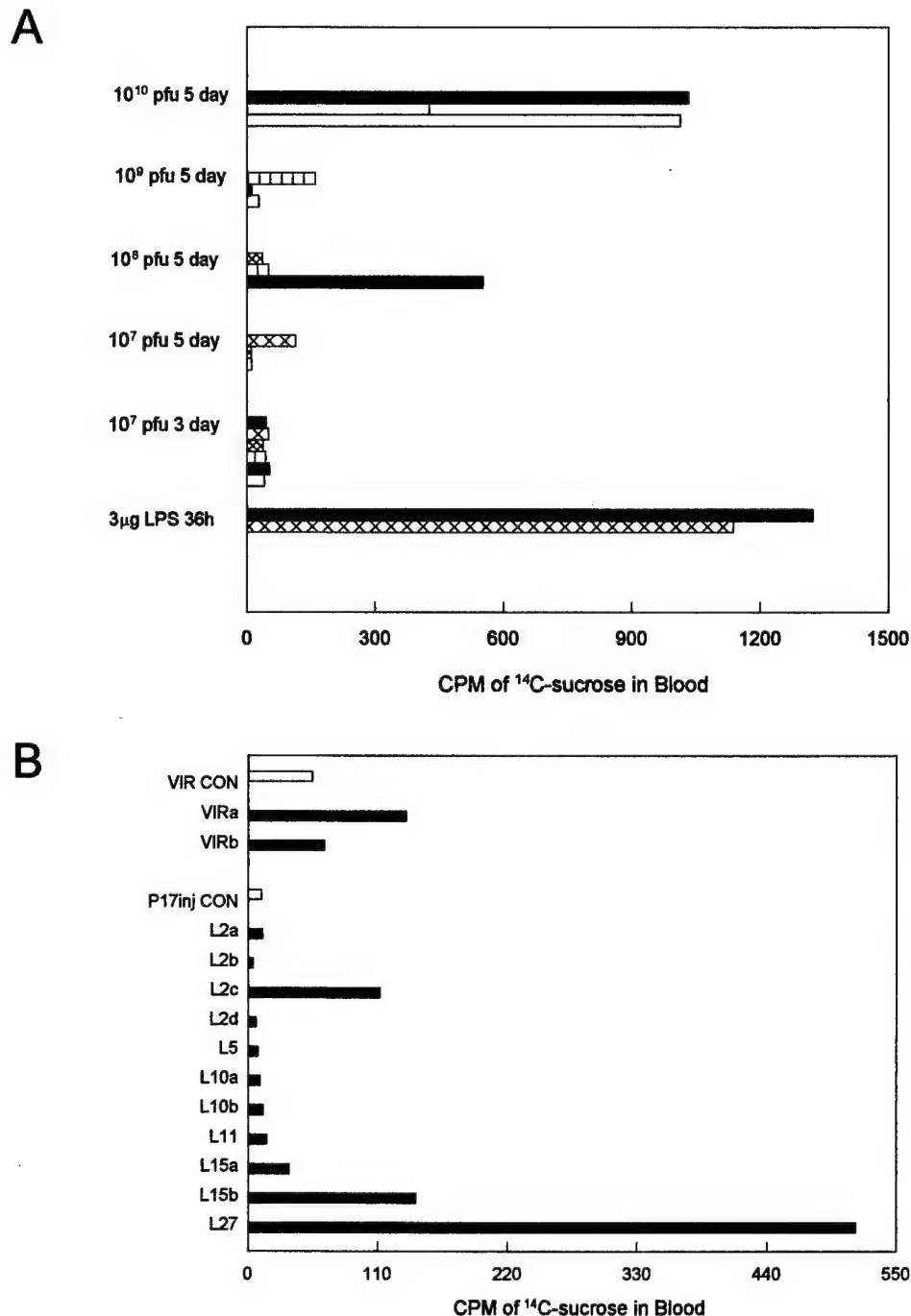
FIG. 1. Epithelial permeability as a function of Ad5GFP dose. (A) Mice were injected at P17 with the stated doses of Ad5GFP, and the permeability of the mammary epithelium was determined by injecting [14C]sucrose intraductally 3 or 5 days postpartum and measuring the amount of 14C in 10 μl of blood. Lipopolysaccharide was injected as a positive control for increased permeability. Each bar represents a different mouse. (B) [14C]sucrose permeability in mice injected at various reproductive stages. [14C]sucrose was injected intraductally into the third mammary glands of nulliparous mice (VIRa and VIRb) and P17 mice at various stages after Ad5GFP injection (see Table 1). Each bar represents an individual mouse. Nulliparous control glands (VIR CON; $n = 5$) received vehicle only. The amounts of [14C]sucrose measured in the blood after injection of P17 control glands (noninjected; $n = 11$) contralateral to Ad5GFP-injected glands (P17inj CON) were averaged.

ganization and 2 represents significant mononuclear cell infiltration and epithelial disorganization. To obtain a mastitis index that provides equal weighting of all three measurements, we used the equation $MI = P/4 + MC + EO$, where MI is the mastitis index, P is the average number of polymorphonuclear cells, MC is the mononuclear cell infiltrate, and EO is the average epithelial organization score. Because 9 was the maximal polymorphonuclear cell count per field, we

chose the value 4 to bring this score in line with the others. Thus, the mastitis score varied between 0 and 6. Three independent observers, one of whom was blinded to the treatment, evaluated each slide with similar results.

Microscopy and quantification of transduction. For an initial assessment of Ad5GFP transduction, the glands were visualized under a Nikon dissecting microscope under fluorescent light. Digital images were captured by using Ax-
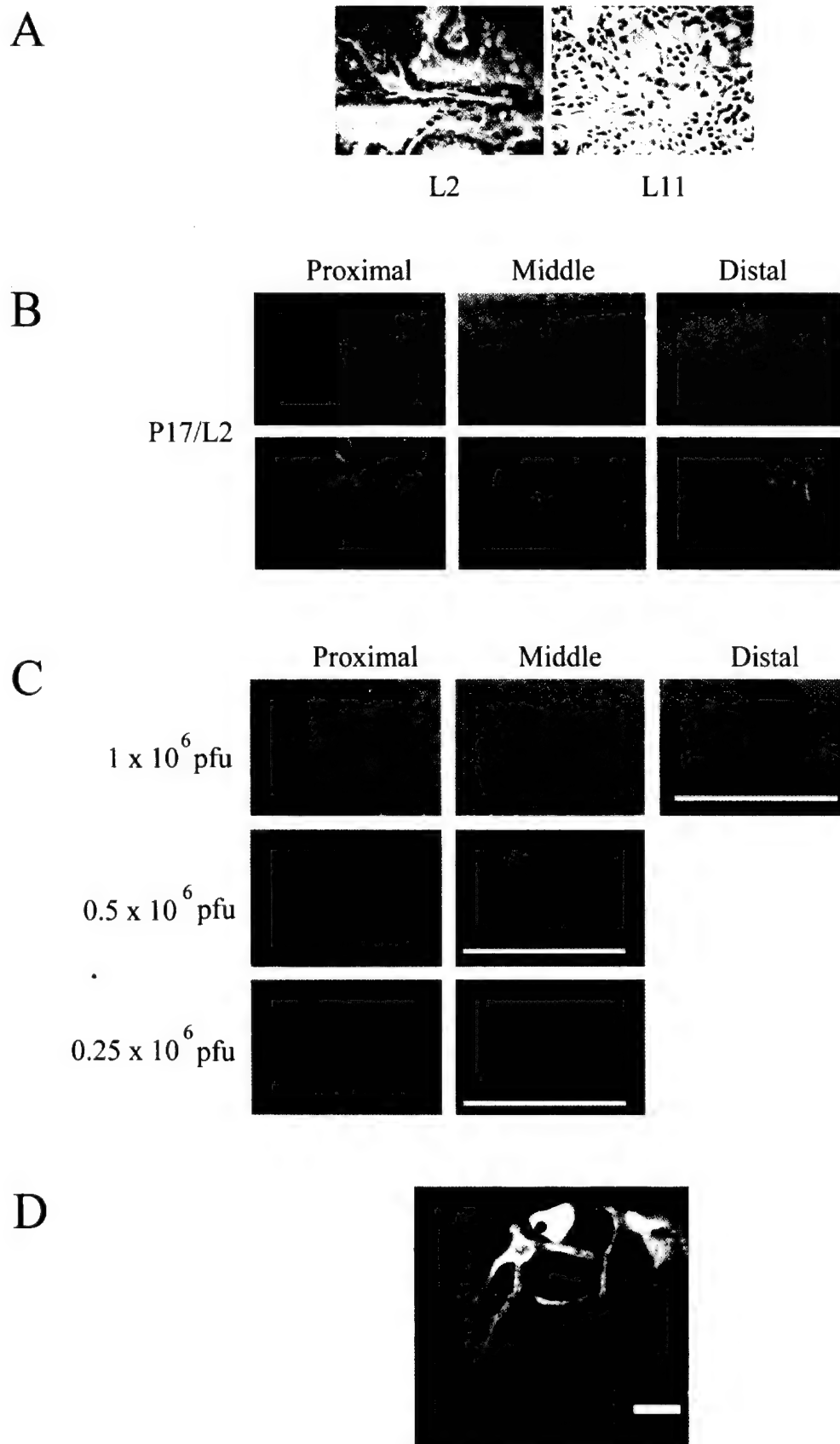
FIG. 2. Histological evidence of mastitis and extent of Ad5GFP transduction in nulliparous animals and animals injected during late pregnancy. (A) Hematoxylin and eosin-stained sections of third mammary glands from mice injected at day 17 of pregnancy (P17) and sacrificed on days 2 (L2) and 11 (L11) of lactation. (B) Initial assessment of transduction in whole glands in regions relative to the teat (proximal, middle, and distal) examined under fluorescent light (upper panel), and 12-μm sections examined under a confocal microscope (lower panel). (C) Whole glands from three individual nulliparous mice injected with different doses of Ad5GFP examined under fluorescent light. Bars, 5 mm. (D) Transduced myoepithelial cell. Bar, 20 μm.
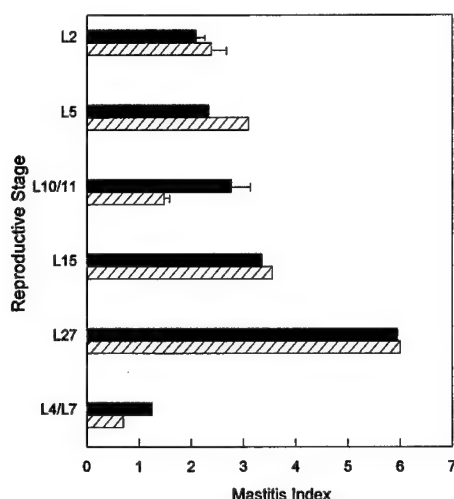
FIG. 3. Evaluation of mastitis. Data represent animals injected with Ad5GFP at P17 and sacrificed at various stages of mammary gland development (solid bars) and their respective controls (noninjected contralateral glands; hatched bars): L2 ($n = 3$), L5 ($n = 2$), L10/11 ($n = 3$), L15 ($n = 2$), L27 ($n = 2$), and L4/L7 ($n = 2$). Glands were assessed at 40× magnification by bright-field microscopy as described in Materials and Methods. See Materials and Methods for definition of the mastitis index.

ioVision software (Carl Zeiss, Inc., North America). A Nikon fluorescent confocal microscope was used to visualize immunohistochemical samples, and Slidebook software (Intelligent Imaging Innovations, Denver, Colo.) was used to capture the higher-power images. Percent transduction of mammary alveoli and/or epithelial structures was determined by manually counting transduced and nontransduced structures in three segments of the gland relative to the teat (proximal, medial, and distal). To determine the extent of transduction of epithelial cells, images were captured at identical magnifications and exposure times and quantified by using a masking program in Slidebook that was capable of determining either the proportion of transduced epithelium or the proportion of transduced nuclei by using DAPI-stained nuclei as a reference.

RESULTS

Adenovirus dosage determination. To determine the maximal dose of adenovirus vector that could be injected without induction of inflammation, various amounts of Ad5GFP were injected intraductally into the fourth mammary glands of P17 mice. We assessed permeability by injecting [^{14}C]sucrose intraductally and measuring its level in the blood (15, 16). Because lipopolysaccharide induces massive mastitis (20), we injected it as a positive control. As the data in Fig. 1A show, high blood levels of sucrose were associated with injection of lipopolysaccharide. An adenovirus dose of 10^{10} PFU also led to high sucrose permeability (Fig. 1A) as well as histological signs of mastitis (data not shown). Sucrose permeability was relatively low with adenovirus doses of 10^8 and 10^9 PFU (Fig. 1A), with the exception of one sample. At a dose of 10^7 PFU, epithelial permeability remained near baseline (Fig. 1A) and the tissue showed no signs of mastitis (data not shown). A dose between 10^7 and 10^8 PFU (2.7×10^7 PFU) was determined to be safe for the fourth mammary gland of the mouse. For experiments in which we used the third gland, which is about one-fifth the size of the fourth gland, we injected 2×10^6 PFU.

Duration of transduction. In the next set of experiments, we used the third mammary gland to assess the amount of damage
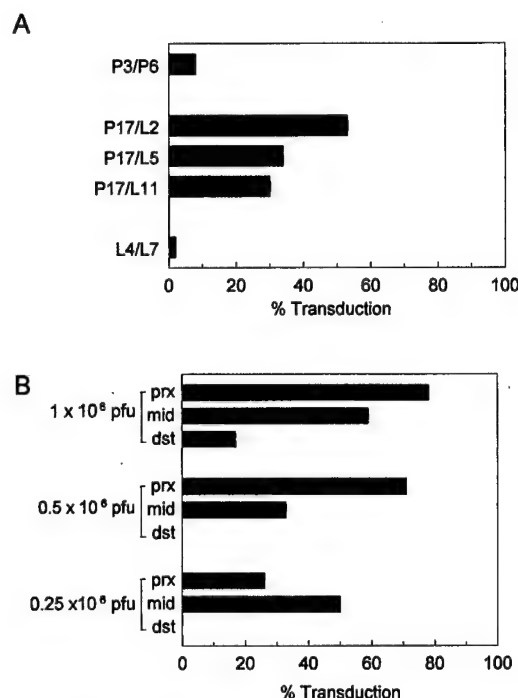

FIG. 4. Percent transduction in early pregnant, late pregnant, and nulliparous glands injected with AdGFP. (A) Percentage of epithelial structures (ducts and alveoli) showing transduction during mammary gland development. Each bar represents an average for three regions of the mammary gland (proximal, medial, and distal relative to the teat) at various stages of development: P3/P6 ($n = 2$), L2 ($n = 3$), L5 ($n = 2$), L10/11 ($n = 3$), and L4/L7 ($n = 2$). (B) Percent transduction of epithelial structures in nulliparous mice at different doses of Ad5GFP. Percentage of transduced epithelial structures was determined manually by counting the number of transduced and nontransduced epithelial structures in nulliparous mice injected with different doses of Ad5GFP: 1×10^6 PFU ($n = 2$), 0.5×10^6 PFU ($n = 2$), and 0.25×10^6 PFU ($n = 1$). Each bar represents the average for three sections in each segment (proximal [prx], medial [mid], and distal [dst] to the teat) of the gland.

incurred by the mammary epithelium at various times after injection in a late pregnant mouse. In some cases, we evaluated the proximal, medial, and distal portions of the gland relative to the teat. As before, intraductal [^{14}C]sucrose injection was used to measure the permeability of the mammary epithelium (Fig. 1B), and mastitis was evaluated in hematoxylin and eosin-stained sections (Fig. 2A). Figure 2A shows an L2 mammary gland with no detectable mastitis. An island of massive mononuclear cell infiltration observed in a gland of a mouse sacrificed on lactation day 11 (L11), 12 days after adenovirus treatment, is also shown in the figure. A mastitis scoring system was designed to provide a semiquantitative measure of the extent of damage. This score, the mastitis index (measured as described in Materials and Methods), is shown for various times after injection in Fig. 3 and, in general, was not different from the index in the contralateral gland The L10 results were confirmed by three observers, but the [^{14}C]sucrose permeability data suggest that the degree of mastitis in the Ad5GFP-injected gland was small. Both the sucrose permeability data and the mastitis score show that an optimal dose of adenovirus produces little damage when injected into late pregnant mice
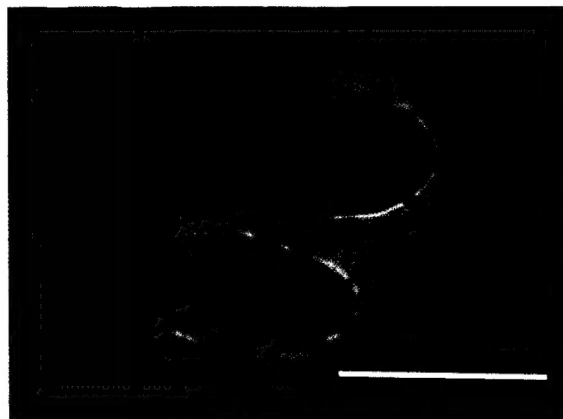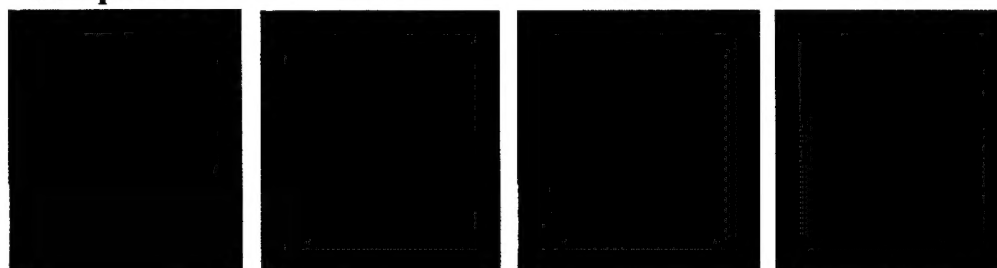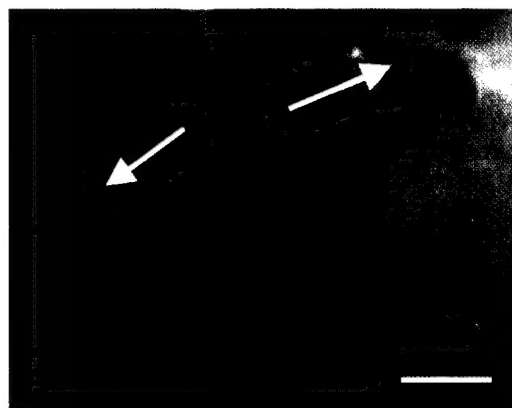
and left up to 5 days or longer into lactation (Fig. 1B and 3). By L27, the gland becomes disorganized due to involutional remodeling (17).

We also examined Ad5GFP transduction in mammary glands from nulliparous mice. We found that volumes larger than 5 μl burst the small ductal system in these glands (data not shown), so all adenovirus injections were restricted to this volume. As a control, 5 μl of sterile-filtered Ringer's solution was injected into the contralateral gland. Three independent observers, one blinded, concluded that there were no gross differences between the control and injected glands and that mastitis appeared relatively minimal (data not shown). The extent of transduction in early pregnant and lactating glands was very low (see below), so mastitis was not formally evaluated in these conditions.

Extent of adenovirus transduction. We viewed transduced mammary glands at low magnification to obtain an initial assessment of the amount of Ad5GFP transduction (Fig. 2B, upper panel, and 2C). Although transduction appeared to be concentrated in areas proximal to the teat or in the middle of the gland in most nulliparous and pregnant animals, some glands did exhibit widespread transduction, as shown in Fig. 2B and 2C (upper panels). We assessed mammary sections from pregnant and lactating mice by using a 20× confocal view of 12-μm-thick frozen sections, first counting the proportion of ducts and alveoli that showed some transduction (Fig. 2B, lower panel). This analysis showed that many but not all alveolar structures were transduced. Less than 10% of the ductal and alveolar structures of glands from early pregnant mice showed some transduction, while approximately 30 to 50% of the structures were transduced when the virus was injected in glands from late pregnant mice (Fig. 4A). Very low transduction was achieved in glands from Ad5GFP-injected lactating mice (Fig. 4A).

To further investigate the extent of alveolar transduction, we used the masking function of Slidebook (see Materials and Methods). By using glands injected at P17 and sacrificed on day 2 of lactation, we were able to delineate specific cellular areas of transduction and calculate the proportion of total epithelium transduced in each alveolar structure. With this approach, we found that the percentage of transduced epithelium was highly variable between two different mice (data not shown). For example, approximately 25% of the total epithelium was transduced at this stage of mammary gland development in areas proximal to the teat in one animal, and only 7% of the total epithelium was transduced in the same area in another mouse. Areas medial and distal to the teat had lower percentages of total transduced epithelium that also varied greatly between animals (data not shown).

To get an approximate measure of the efficiency of transduction, an estimation of the number of epithelial cells in the third mammary gland was made and compared with the frequency of transduction. By using the DNA content of the gland as a starting point, it was estimated that there were approximately 3×10^7 epithelial cells. Approximately 20%, or 6×10^6, of these cells were transduced in the most highly transduced gland when 2×10^6 PFU of Ad5GFP were injected. The ratio of transducing units to PFU was near 5:1, so approximately 10^7 transducing units were injected. This analysis suggests that greater than half of the injected vector transduced epithelial cells, supporting the idea that the process is relatively efficient during the late stage of pregnancy in the mammary gland.

The mammary gland consists of an inner layer of ductal cells and alveoli and an outer contractile monolayer of myoepithelial cells closely attached to the basement membrane. The myoepithelial cells extend laterally along ducts and form a basket-like sheath around both ducts and alveoli (13). In response to oxytocin binding to specific receptors, myoepithelial cells contract and expel milk from the alveoli into the ducts and eventually out of the gland. The presence of GFP in myoepithelial cells in glands transduced in late pregnancy (Fig. 2D) suggests that viral particles were able to traverse the paracellular compartments of the mammary gland or that some myoepithelial cells were exposed at the luminal surface. The presence of GFP in myoepithelial cells also raises the possibility that the basolateral surface of the mammary epithelium is accessible during late pregnancy, when the paracellular spaces are open to large molecules, allowing adenovirus access to its receptor (1, 23, 27).

Epithelial structures of nulliparous mice can also be transduced by Ad5GFP (Fig. 2C and 4B). Portions of the gland proximal to the teat were highly transduced after injection with relatively high doses of virus, but the extent of transduction was greatly reduced with decreasing doses (Fig. 2C and 4B). It is possible that adenovirus transduction can be used experimentally in nulliparous animals with small volumes and careful attention to optimizing the dose.

Functional competence of transduced cells. The data presented to this point show that mammary epithelial cells can be transduced with Ad5GFP during pregnancy and can be maintained well into lactation without inflammation. However, in order to utilize adenovirus microinjection as an effective method of changing gene expression, transduced cells must retain functional integrity. Two distinct pathways of cellular milk synthesis, milk protein secretion and milk fat secretion, can be assessed morphologically. Transduced alveoli displayed normal morphology, produced and secreted milk fat globules, and were laden with milk, which was stained red in the lumen of the alveoli in Fig. 5A. Casein, a milk protein, was also produced and was detected in the lumen of transduced alveoli (Fig. 5B). Xanthine oxidase has been shown to redistribute from the cytoplasm to the surface of emerging milk fat globules during pregnancy at the onset of lactation, and this redistribution is thought to be essential for milk fat globule release (J.

FIG. 5. Maintenance of function in mammary glands transduced with Ad5GFP. (A) Two nearly completely transduced (green) alveoli showing milk oligosaccharides stained with rhodamine-wheat germ agglutinin (red), nuclei stained with DAPI (blue), and surrounding milk lipid droplets. These alveoli appear morphologically normal. Bar, 100 μm. (B) A 20× view of lumens of transduced (green) alveoli stained with anticasein antibody (red); the nuclei were stained with DAPI (blue). (C) A 20× and (D) a 100× view of fat droplets in transduced (green) alveoli rimmed with xanthine oxidase (XO) as shown by stain (red) with an anti-xanthine oxidase antibody; the nuclei are stained with DAPI (blue). Arrows indicate milk fat droplets rimmed with xanthine oxidase in panel D. Bar, 20 μm.

McManaman, personal communication). This localization of xanthine oxidase was maintained in transduced cells (Fig. 5C and 5D). Thus, the transduced cells appeared to be fully functional.

DISCUSSION

Adenovirus vectors have been used to transduce a variety of organs in a number of animal species. Mice have been especially popular as models to study the effects of adenovirus vectors. Intraductal injection of adenovirus vectors provides a versatile method of altering gene expression in both the luminal and, to some extent, the myoepithelial cells of mouse mammary glands. In no case did we find transduction in the interstitial spaces of the mammary glands that we studied. It is possible to transduce nulliparous glands (Fig. 2C and 4B), even though these glands are immature compared to late pregnant glands and are particularly smaller in size. Although adenovirus can be used to transduce the epithelium at any stage of mammary gland development except lactation, the technique has proven to be most effective during the switch from pregnancy to lactation (Fig. 2B, 3, 4A, and 5A). Little mastitis was observed up to a week to 10 days after transduction with an optimal dose of virus (Fig. 1 and 3). The extent of transduction was highly variable within regions of the gland relative to the teat and may not generate enough material for biochemical analysis of transduced cells. Nevertheless, morphological studies may still be possible with neighboring nontransduced cells as controls.

We had less success with adenovirus transduction at other stages of mammary gland development. Transduction during early pregnancy was relatively low (Fig. 4A), possibly because the epithelium is turning over rapidly at this developmental stage (2). Transduction during lactation (Fig. 4A) was rarely successful. Our laboratory has previously shown that tight junction permeability is very low during lactation (16), preventing solute passage through the paracellular pathway to the basal surface of the gland, where the adenovirus receptor may be localized (23, 27) There is also evidence that the coxsackie and adenovirus receptor is a component of the tight junction complex and that its localization within this complex impedes viral transduction (3).

Taking these factors into consideration, it is possible that the change in tight junction permeability that accompanies secretory activation is responsible for the low efficacy of adenovirus transduction during lactation. Also, during lactation, the high concentration of milk proteins may adsorb the viral particles. The density of adenovirus receptors may also differ at different stages of mammary gland development with changes in endocrine state, and these differences may influence the efficacy of adenovirus transduction (7). Thorough investigations with such techniques as in situ hybridization and adenovirus receptor density studies in mouse mammary glands at various stages of development are needed to properly address these issues. Nevertheless, adenovirus vectors appear to be effective in mediating the transduction of functional genes into mouse mammary epithelial cells, particularly ain late pregnancy, when the tight junctions between epithelial cells are open to the passage of large molecules (15).

Our results also demonstrate that transduction with Ad5GFP does not disrupt normal mammary epithelial cell morphology and function (Fig. 5). Adenovirus transduction did not disrupt either of two major and distinct synthetic pathways for cellular milk secretion, milk protein secretion and milk fat globule formation. These data clearly show that adenovirus transduction can be used to alter gene expression and to study luminal cell function in the mouse mammary epithelium.

Adenovirus vectors used in sufficient amounts to efficiently transduce target tissues generally induce a strong inflammatory response that typically reaches a high level within 4 to 7 days of vector introduction in a variety of immunocompetent mouse strains. Inflammation occurs within targeted organs transduced via the bloodstream, such as the liver (25), as well as when the mucosal surface of an organ is targeted, such as in the lung (26). The inflammatory response both limits the duration of expression and leads to an immune response that limits successful reuse of the vector. The relatively rapid induction of inflammation has limited the usefulness of adenovirus vectors for studies of altered gene expression in most tissues. Thus, the findings presented here are somewhat surprising.

It is not clear why the induction of inflammation is delayed when adenovirus vectors are used in sufficient amounts to transduce a significant fraction of the mammary epithelium. It is possible that this delay is due to the use of outbred CD-1 mice. Adult CD-1 mice have been successfully transduced for extended periods of time with adenovirus vectors (22), and neonatal CD-1 mice did not exhibit inflammation after injection with an adenovirus vector (21), raising the possibility that CD-1 mice may exhibit reduced inflammatory and immune responses to adenovirus vectors relative to other mouse strains. Alternatively, it may be that pregnancy reduced the immunocompetence and inflammatory response of the mice. Regardless, the delayed onset of inflammation means that adenovirus vectors can be effectively used as a tool for altering gene expression in the mammary gland of CD-1 mice.

The usefulness of first-generation adenovirus vectors in transduction of the mammary epithelium of late pregnancy means that it is not necessary to undertake the substantial efforts required for the use of helper-dependent, or "gutless," vectors. However, it remains a possibility that the use of second-generation vectors made replication incompetent through deletion of a viral gene essential for viral DNA replication, such as the terminal protein gene (11), will lead to a reduction in the low level of inflammation or extend the time prior to the appearance of inflammation, extending their usefulness. Since such vectors are relatively easy to construct and grow, it may be worthwhile to test their effectiveness in transducing the mammary epithelium.

Intraductal microinjection of adenovirus vectors should aid in studies of a variety of genes of interest in the mammary epithelium. Since adenovirus transduction appears to be confined to the mammary epithelium, these methods provide a technique to target genes of interest to this tissue compartment. Potentially, these techniques could be used for drug, hormone, or protein delivery to milk on a short-term basis. Currently, our laboratory is preparing to use the methods described here to examine the regulation of milk synthesis and secretion in the mouse mammary epithelium. Recombinant adenovirus constructs could potentially target genes responsible for certain types of breast cancers. The procedures de-

scribed here provide a means of studying the efficacy of such vectors.

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